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Article (Accepted Version)

Souissi, Wided, Kaloki, Arnold, Etherington, Stuart, Domanska, Barbara, West, Michelle J and Crickmore, Neil (2019) Differential proteolytic activation of the *Bacillus thuringiensis* Cry41Aa parasporin modulates its anticancer effect. *Biochemical Journal*, 476 (24). pp. 3805-3816. ISSN 0264-6021

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Differential proteolytic activation of the *Bacillus thuringiensis* Cry41Aa parasporin modulates its anticancer effect

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Abstract

Bacillus thuringiensis (Bt) is a gram positive spore forming bacterium which produces intracellular protein crystals toxic to a wide variety of insect larvae and is the most commonly used biological pesticide worldwide. More recently, Bt crystal proteins known as parasporins have been discovered, that have no known insecticidal activity but target some human cancer cells exhibiting strong cytotoxic activities with different toxicity spectra and varied activity levels. Parasporin-3, also called Cry41Aa, has only been shown to exhibit cytotoxic activity towards HL-60 (Human promyelocytic leukemia cells) and HepG2 (Human liver cancer cells) cell lines after being proteolytically cleaved. In order to understand this activation mechanism various mutations were made in the N-terminal region of the protein and the toxicity against both HepG2 and HL-60 cell lines was evaluated. Our results indicate that only N-terminal cleavage is required for activation and that N-terminally deleted mutants show some toxicity without the need for proteolytic activation. Furthermore we have shown that the level of toxicity towards the two cell lines depends on the protease used to activate the toxin. Proteinase K-activated toxin was significantly more toxic towards HepG2 and HL-60 than trypsin-activated toxin. N-terminal sequencing of activated toxins showed that this difference in toxicity is associated with a difference of just two amino acids (serine and alanine at positions 59 and 60 respectively) which we hypothesize occlude a binding motif.

Keywords: Cry toxin, HepG2, HL-60, activation

Abbreviations: FL: full length, PK: proteinase K, try: trypsin. MS-grade: Mass spectrometry-grade PVDF: Polyvinylidene difluoride, Pres: PreScission, sol: solubilised, CH: chymotrypsin, BBMV: brush border membrane vesicles, Sol: solubilized, FP: forward primer, RP: reverse primer, aa: amino acids

Introduction

Bacillus thuringiensis synthesizes crystalline parasporal inclusions during sporulation and this salient feature makes it distinguishable from other *Bacillus* species [1]. The inclusion proteins have been proven to be highly toxic to insects making Bt an entomopathogenic organism that has been widely used as a biological pesticide in the form of sprays and more recently Bt proteins have been expressed in transgenic plants rendering them resistant to insect attack [2]. Bt strains show activity towards larvae of diverse insect orders including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga and in some cases against species from other phyla like nematodes, mites and protozoa [3]. In addition, more recently discovered Bt toxins, named as parasporins, were shown to exhibit activity against human cancer cells of various origins [1]. Amongst these parasporins, parasporin-3 most closely resembles the commercially used insecticidal toxins and presents the narrowest activity spectrum, showing moderate cytotoxicity against only two of the tested cancer cell lines, HL-60 (Human promyelocytic leukemia cells) and HepG2 (Human liver cancer cells).

Processing of Cry protoxin into its active form is essential for toxin activity and this processing is mediated by proteases that cleave the protoxin polypeptide at specific points to produce the mature active toxin. In the case of parasporins, proteolytic cleavage was also shown to be indispensable for toxin activity [1]. Previous work on Cry41Aa had shown that solubilised protein had no cytotoxic activity against HepG2 cells whereas upon treatment with proteinase K it exhibited cytotoxic activity against both HepG2 and HL-60 cell lines [4]. It was shown that proteinase K cleaves the 88 kDa protein after amino acid 60, leading to the production of a major band of 64 kDa which was identified as being the toxic moiety. A more recent study was in agreement with these findings showing that upon proteolytic treatment with trypsin a 66 kDa protein was produced that induced a rapid and significant decrease in HepG2 metabolic activity [5]. The loss of ca 24 kDa upon activation, and the identification of the N-terminal site at amino acid 60 indicates that there is also C-terminal cleavage. The lack of a role for the C-terminal region in toxicity was confirmed by the deletion of this part of the toxin-encoding gene [5]. Earlier work on insecticidal toxins has suggested a number of roles for N-terminal activation including the revealing of a binding epitope [6] and as a membrane insertion trigger [7]. In this work we further investigated the role of proteolytic activation in the mechanism of action of Cry41Aa and established that only cleavage at the N-terminus is required to produce a functional protein. Importantly we observed that the use of different proteases can modulate the activity of the toxin.

Experimental procedures

Creation of mutant toxins

Inverse PCR was used in order to create the desired mutations. The primers utilized are presented in table 1. Confirmation of the mutations created was carried out using DNA sequencing (Eurofins Genomics).

Crystal protein harvesting and activation

Bt transformants were grown on LB agar plates containing 5 µg/ml chloramphenicol for 3 days at 30°C. Sporulation and production of crystals were monitored using a phase contrast microscope. The sporulated cells and crystals were scraped off the plate and sonicated. Crystal proteins were then solubilised in 50mM sodium carbonate at pH 10.5 in the presence of 5 mM Dithiothreitol (DTT) at 37°C for 1 hour. Supernatant was collected and treated with the appropriate protease. Following addition of trypsin (1 mg/ml final concentration) or proteinase K (0.001 mg/ml final concentration) the mixture was incubated at 37°C for 1 hour while for activation with PreScission protease (130 µg/ml), sample was incubated at 4°C for 16 hours. Complete mini EDTA-free protease inhibitor (Roche) was finally added to the activated samples to stop further proteolysis.

Protein purification

Purification of crystal proteins was carried out using a 1 ml Resource Q column (GE Healthcare Life Sciences) connected to an ÄKTA Purifier-FPLC System. Samples were injected in 10 mM CAPS (pH 10.5) and a linear increase in the gradient of NaCl (0 to 1 M) was applied at a flow rate of 1 ml/min for 25 min. Protein concentration was determined by the Bradford method with a Bio-Rad Protein Assay Kit using bovine serum albumin as the standard.

Cell culture

HepG2 and HL-60 cell lines were purchased from the European Collection of Cell Cultures (ECCAC; Salisbury, UK). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) and in Roswell Park Memorial Institute medium (RPMI) 1640 respectively, provided with 1% penicillin-streptomycin-neomycin (PSN) antibiotic mixture and 10% fetal bovine serum (FBS), in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were split when they reach 70-80% confluence.

Cell assays

Assays were performed in 96-well plates (Nunc). Each well received 90 µl of cell suspension at a density of 22,500 cells per well and cultured overnight (at 37°C/5% CO₂ humidified air) before 10 µl of the test sample was added. The experiments were set up in triplicate.

The mock control wells received 90 µl of cell suspension and 10 µl of the appropriate buffer (Na₂CO₃ or PBS). 90 µl of cell suspension and 10 µl of Triton X-100 (0.01%) containing wells served as a positive control while the wells that contained 100 µl of appropriate cell culture medium served as background fluorescent/luminescent controls.

The readings were carried out using GloMax-Multi Detection System (Promega) according to the assay instructions. The fluorescent or luminescent signal in the background control wells was subtracted from each experimental value. Error bars represent one standard error of the mean.

Results

N-terminal deletions of Cry41Aa

Although trypsin [5] and proteinase K [4] both resulted in proteins of circa 65kDa we decided to establish the actual trypsin cleavage site. Cry41Aa was activated with trypsin (MS-grade) and subjected to N-terminal sequencing. This revealed an N-terminal sequence of YPFAQ representing a cleavage after R23. To confirm that cleavage here could result in an active toxin a deletion of amino acids 2-23 from the full length (FL) protoxin was created. The mutated protein was named FL Δ 23 and successfully expressed (Fig 1). Note that the circa 100kDa band observed in the crude sample represents the previously described (add Krishnan Ref) ORF3 crystallization factor.

The level of toxicity of solubilised FL Δ 23 was then evaluated against HepG2 (Fig 2). At a concentration of 100 μ g/ml, neither solubilised FL Δ 23 nor solubilised protoxin had any significant effect on cells. In contrast a much lower concentration of trypsin activated Cry41Aa (12 μ g/ml) caused a considerable decrease in cell viability.

In an attempt to understand why the toxin activated by trypsin at amino acid 23 was active whilst the toxin with these amino acids deleted was not, we tested the protein sent for sequencing (activated by MS-grade trypsin) for HepG2 activity and surprisingly found no significant activity, in contrast to toxin activated by our lab-grade trypsin (Fig 3).

To test for possible differences between the two grades of trypsin we then sent some toxin, activated by lab-grade trypsin, for sequencing. This revealed an N-terminus of SADVR indicating a cleavage after Y58. Based on this finding we concluded that our lab-grade trypsin contained another protease, most likely chymotrypsin, that could cleave after Y58 and result in toxin activation. To test this Cry41Aa was treated with chymotrypsin and found to have activity against HepG2 cells (Fig 4).

In the rest of this work we shall continue to refer to our lab-grade trypsin as trypsin although it is likely that the functional proteinase is most probably chymotrypsin. We also decided to continue to use our lab-grade trypsin for activation studies, rather than switch to chymotrypsin, to provide consistency with our previous studies. We decided to then delete the first 60 amino acids to better represent proteolytically cleaved toxin. FL Δ 60 was created however it had a very poor expression and was not stable after proteolytic treatment (data not shown). This could be explained by a possible role of the deleted peptide in protein folding. Since the deletion in FL Δ 23 was not sufficient to produce an active form of the toxin, and since FL Δ 60 was not properly expressed/folded we attempted to create a pre-activated toxin by deleting 40 amino acids (we felt that deleting 58 amino acids would most likely result in the same problem as deleting 60). The mutated protein FL Δ 40 was successfully created and although its expression was also poor, it did appear stable. The cytotoxic effect of FL Δ 40 was then assessed on HepG2 cells (Fig 5) and showed that at a concentration of 10 μ g/ml, solubilised FL Δ 40 had higher toxicity compared to solubilised FL at a concentration of 100 μ g/ml. Therefore N-terminal cleavage at amino acid 40 appears to be sufficient for Cry41Aa toxicity. However solubilised FL Δ 40 showed reduced toxicity compared to trypsin

activated Cry41Aa raising the question as to whether C-terminal cleavage, or N-terminal cleavage beyond amino acid 40, may play a role in toxicity.

The role of C-terminal cleavage in Cry41Aa activation

As well as the circa 65 kDa produced upon proteolytic activation of Cry41Aa both Yamashita et al [4] and ourselves had observed (Fig 6) a higher molecular weight band, when activated with trypsin or proteinase K, that is slightly smaller than the full-length toxin and represented a partial digest. Yamashita et al had shown that the lower band was active but we were interested in knowing whether C-terminal cleavage was important for toxicity, or affected the level of toxicity. To do this we attempted to chromatographically separate the two bands.

Separation by gel filtration resin Sephacryl S-200 was unsuccessful since the two fragments co-eluted (data not shown). Anion exchange chromatography was then used which allowed us to successfully separate the upper, but not lower, band from the mixture (Fig 6). Figure 7 shows that there was no significant difference in toxicity between the upper band and the mixture, which indicates that C-terminal cleavage is not required for toxicity.

To completely rule out any role of the C-terminal region in toxicity we decided to create a toxin variant that could only be cleaved at the N-terminus. To do this we engineered the highly specific PreScission protease recognition site into the toxin to allow cleavage after amino acid 40 (FLP40) (Fig 8).

Figure 9 shows that a single band was produced when activated by PreScission that was similar in size to the upper, higher molecular weight, band produced by trypsin, but smaller than the uncleaved toxin indicating that cleavage had occurred (Fig 9A). A Western blot was also performed (data not shown) which confirmed that a C-terminal tag was still present following cleavage. The level of toxicity of this activated FLP40 was then evaluated on HepG2 and showed that it had a similar level of activity to trypsin activated toxin (Fig 9B). This confirmed that Cry41Aa toxin could be activated with PreScission protease and therefore that N-terminal activation alone is sufficient for toxicity.

Effect of different proteases on the activity of Cry41Aa

Having shown previously [5] that trypsin activated Cry41Aa showed good activity against HepG2 but not against HeLa cells we wanted to confirm the findings of Yamashita et al that this toxin also had activity against HL60 cells. We activated the toxin with both trypsin and proteinase K and our cytotoxicity results showed that proteinase K (PK) activated Cry41Aa was significantly more toxic to HL60 than trypsin activated toxin (Fig 10).

For HepG2 PK-activated toxin was more active, although the difference was much less dramatic. Having confirmed above that our lab grade trypsin cleaved Cry41Aa after amino acid 58 we wanted to confirm the previous finding that proteinase K cleaved the toxin after amino acid 60 [4]. N-terminal sequencing gave a sequence of DVRDA confirming that cleavage site. Although we had ruled out C-terminal cleavage as being necessary for toxin activity, given just the two amino acid difference at the N-terminus we wanted to establish

whether differences in C-terminal cleavage could help explain the difference between the trypsin and proteinase K activated Cry41Aa. To do this we first checked whether the upper band of the activated protein was getting cleaved. To achieve that we added a C-terminal HA tag to the toxin and then used Western Blotting to establish whether the tag was still present. We established that the tag was still present on the protein (upper band) regardless of which protease was used to activate (data not shown). This confirmed that there was no C-terminal cleavage in the upper band and so these upper bands were purified by anion-exchange chromatography and used for cytotoxicity assays. The results from these assays (Fig 11) mirrored those described above (Fig 9B) demonstrating that C-terminal cleavage was not involved in the differential activity.

Modulation of Cry41Aa activity through differential N-terminal cleavage.

The data described above clearly point to the fact that just a 2 amino acid difference in the N-terminal cleavage site can have significant effects on the activity of Cry41Aa, particularly against HL60. In order to test this we endeavoured to introduce a trypsin cleavage site after amino acid 60 – by creating an A60K mutant – to see whether this mutant, when activated with trypsin, would behave the same as the non-mutant when cleaved, at the same location, by proteinase K. The mutant was successfully created and expressed, however analysis of the activated mutant indicated that it had not been cleaved by MS grade trypsin at amino acid 60 but just at amino acid 23 (data not shown).

Discussion

The processing of Cry protoxins has been well described. Cleavage of these proteins usually results in the removal of N-terminal peptides, and often C-terminal peptides (from the larger protoxins). We have shown that the N-terminus of the protein plays an important role in protein expression/crystallization. Deletion of 40 amino acids at the N-terminus led to weak expression of the protein and deletion of 60 resulted in very poor protein expression. Previous research with mutant toxins indicated that toxin stability and or/expression levels are affected by N-terminal sequences. For example, expression of Cry1C toxins with truncated N-termini were not expressed as effectively as those with an intact N-terminus [8] [9].

In addition to its role in expression, it has previously been shown that N-terminal cleavage is essential for toxin action. This is the case for both insecticidal and anti-cancer Cry toxins. According to our results N-terminal cleavage alone was sufficient to activate Cry41Aa. This was shown by the use of FLP40 which was cleaved only after amino acid 40 and was active against HepG2 cells. The fact that an N-terminal deletion (FLΔ40) was also active supported this finding. Previous studies have shown that cleavage of the N-terminus of parasporin-2 toxin was essential for its cytotoxic activity against MOLT-4 cell line [10]. Parasporin-1 (81 kDa) was also shown to be only toxic to cancer cells after being proteolytically activated at the N-terminal region resulting in the production of an active form consisting of 15 and 56 kDa polypeptides [11]. In the case of insecticidal toxins, lack

of cleavage at the N-terminus of a variant of Bt Cry1Ac by trypsin made it unable to form pores in vitro in *Manduca sexta* brush border membrane vesicles and had reduced insecticidal activity in vivo [7].

In contrast, it has been shown that the carboxy-terminal extensions of many Cry toxins have no major role in their activity. Deletion of the C-terminal sequence of Cry15Aa for example showed that this sequence is not required for activity against the codling moth (*Cydia pomonella*) [12]. Schnepf et al in 1998 reported that in *Bacillus thuringiensis* subsp. *kurstaki* HD-1, deletions up to the 645th codon at the 3' end allowed synthesis of the toxic peptide. These deletions made at the C-terminal region of the protein were shown to have no effect on toxin activity [3].

Since activation is a crucial step to achieve toxicity, it has been suggested that the type and/or abundance of proteases is important in contributing to toxin specificity. Our results showed that the toxic effect of Cry41Aa towards HepG2 and HL60 cell lines depends on the protease used to activate the toxin. This result agrees with previous studies where the type of protease used was important in Cry toxicity/specificity. The insecticidal specificity of Cry toxin from *B. thuringiensis* var. *colmeri*, serotype 21, was influenced by the species of insect used to provide gut enzymes for activation. The activation of the toxin with different gut extracts produced different sizes of toxins that showed different specificities. Protoxin that was shown to have insecticidal activity against Lepidopteran and Dipterian larvae, only killed *A. aegypti* when activated with *A. aegypti* gut extract while it was toxic to both *Pieris brassicae* and *A. aegypti* larvae when treated with *P. brassicae* gut extract [13]. Reasons behind this difference in toxicity were suggested in previous research studies. Activation of Cry3 protoxin with different proteases was shown to affect toxin function since chymotrypsin but not trypsin activated Cry3Ba was able to bind to BBMV of Colorado potato beetle *Leptinotarsa decemlineata*. The loss of binding of trypsin activated Cry3Ba toxin was suggested to be due to structural changes related to proteolysis or to processing of important binding epitopes in the toxin [14]. In case of anticancer Cry toxins, it has been shown that only upon treatment with proteinase K or trypsin is parasporin-1 toxic to MOLT-4 cells while no cytotoxic activity was observed following chymotrypsin treatment. It was suggested that this could be due to the fact that same proteolysis profile was produced by treatment with trypsin and PK while chymotrypsin activation gave a different protein banding pattern where a band of 56 kDa was not produced [15]. This 56 kDa protein was later shown to form a part of a heterodimer believed to be the toxic moiety [11].

The importance of the position of N-terminal cleavage has also been previously highlighted. The introduction of a chymotrypsin/cathepsin G site in the loop between helix α -3 and helix α -4 of Cry3A toxin increased its toxicity 3 fold against neonate *D. virgifera* larvae. The processing of this toxin was shown to be more rapid which is likely due to the introduced chymotrypsin/cathepsin G site being more exposed for interaction with the enzyme than the native chymotrypsin site. This allowed faster and specific binding to the insect BBMV. Hence, it has been proposed that cleavage of the toxin at this proteolytic site permitted the subsequent binding of the activated toxin to the receptors present in the midgut cells [16]. Bravo et al [7] suggested that the presence of the N-terminal peptide might prevent binding to insect gut membranes which only occurs after proteolytic removal of this peptide. The creation of an N-terminally deleted variant of Cry2A toxin, which resulted in a 3 fold increase in its toxicity against *Spodoptera littoralis* and *Agrotis ipsilon*, suggested that the removal of 42 amino acids serves to expose the receptor-binding region of the toxin and that this amino terminal cleavage is a rate-limiting step in the toxin binding of Cry2A [17]. This was supported by the fact that the structure of Cry2Aa revealed that the N-terminal

region masks a region of the toxin proposed to be involved in the interaction between the toxin and the brush border membrane of the target insect [6].

In the case of parasporin-2, no cytotoxic activity was observed after trypsin treatment of solubilised crystal proteins whereas proteinase K activated proteins were highly cytotoxic to HepG2, MCF-7, KLE, Hec-1A, MDA-MB231 and PC-3 cells [18]. It was suggested that the difference in toxicity between the trypsin and PK activated toxins was directly related to the different cleavage sites. They hypothesized that without proper protease activation, the PS2Aa1 protoxin could not be recognized by cell receptors. It was assumed that when cleaved with proteinase K, specific regions of the activated PS2Aa1 can bind to a receptor while, when cleaved with trypsin the binding is prevented because these binding epitopes can only be partially exposed or not at all. Based on the predicted structure of Cry41Aa the two extra amino acids present following trypsin activation (S59, A60), compared to PK activation, potentially could be partially covering the N-terminal sequence of PK activated toxin (Fig 12). The fact that deletion of 40, but not 23, amino acids at the N-terminus produced an active toxin that was capable of killing HepG2 cells supported the fact that the position of N-terminal cleavage can have an effect on toxin activity. Therefore the hypothesis is that the N-terminal region of Cry41Aa may be interfering with binding and deletion of the first 23 amino acids was not sufficient to expose the binding site of Cry41Aa whereas deletion of 40 led to at least some exposure of this region.

Acknowledgement:

This work was partially funded through Bloodwise grants 15004 and 15024 and Medical Research Council grant MR/S009620/1 to MJW.

Conflict of interest:

We have no conflicts of interest to disclose.

Author contributions:

W.S.; conceive and conduct the experiments, S.E.; Creation of a tagged Cry41Aa that was used as a control (data not shown), A.K.; Creation of A60K mutant, B.D.; initially characterised the differential activation of Cry41Aa against HepG2 cell line, M.W.; provided tissue culture facilities and advised, N.C.; supervision, review and editing.

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Tables:

Table 1: List of mutants created and their correspondent primers and template used.

Mutant created	Template	Forward and reverse primers (5' —————> 3')
FLΔ23	SVP2741Aa	FP: TATCCTTTTGCGCAGGCAC RP: CATTGACAATCCTCCATTCCA
FLΔ40	SVP2741Aa	FP: GAGTGGATGAATATGTGTACTAGTGG RP: CATTGACAATCCTCCATTCC
FLΔ60	SVP2741Aa	FP: GATGTAAGGGATGCCGTTATTAC RP: CATTGACAATCCTCCATTCCA
FLP40	SVP2741Aa	FP: TTTCAAGGTCCTATGAATATGTGTACTAGTGG RP: AAGTACTTCAAGTAGTTCAGAACCTGGTGC
FL-HA	SVP2741Aa	FP: TTCCAGATTATGCTTAAAGGTGTGCAACTATC CCTG RP: CATCATATGGATAAGTGGTTAAGCCAATACCCA TAC
AK	SVP2741Aa	FP: AAAGATGTAAGGGATGCCGTTATTAC RP: GCTGTATCCCTCCCCCAG

Figures

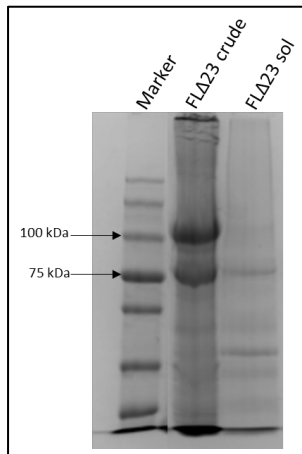


Figure 1: SDS-PAGE analysis of FLΔ23 protein levels.

Crude (spores and crystals mixture) FLΔ23 was solubilised for 1 hour at 37°C in 50 mM sodium carbonate (pH 10.5) in the presence of 5 mM DTT. The samples (crude and solubilised) were then run on 7.5% SDS-PAGE gel.

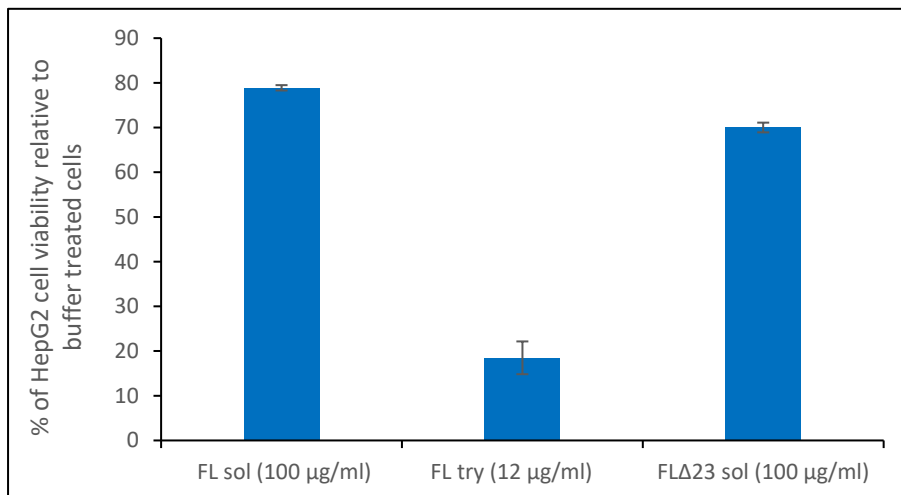


Figure 2: Evaluation of the level of toxicity of solubilised FLΔ23.

HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with solubilised FL (100 µg/ml), solubilised FLΔ23 (100 µg/ml) and trypsin activated FL (12 µg/ml). 24 hours later, cell viability was measured using CellTiter-Blue.

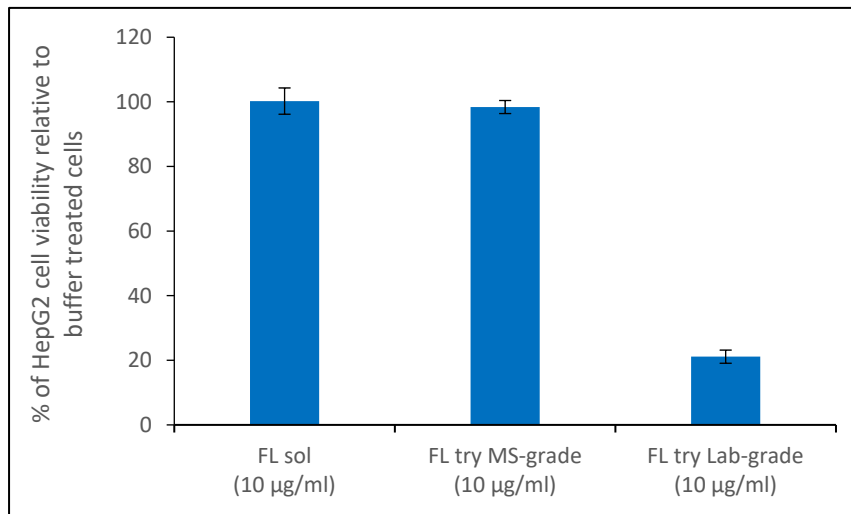


Figure 3: Assessment of the level of toxicity of Cry41Aa activated with MS-grade vs Lab-grade trypsin.

HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with a concentration of 10 µg/ml of solubilised Cry41Aa (FL sol), activated Cry41Aa with pure MS-grade trypsin (FL try MS-grade) and activated Cry41Aa with lab-grade trypsin (FL try Lab-grade). 24 hours later, cell viability was measured using CellTiter-Blue.

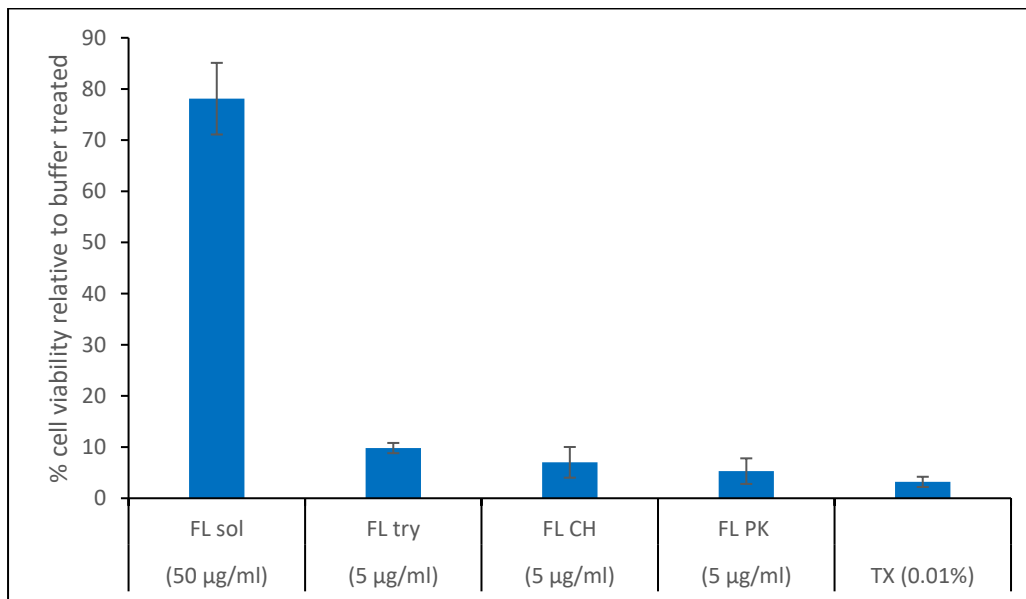


Figure 4: Evaluation of the cytotoxic effect of chymotrypsin activated Cry41Aa.

HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with solubilised FL (50 µg/ml), trypsin, chymotrypsin and proteinase K activated FL (5 µg/ml) and Triton-X (0.01%). 24 hours later, cell viability was measured using CellTiter-Blue.

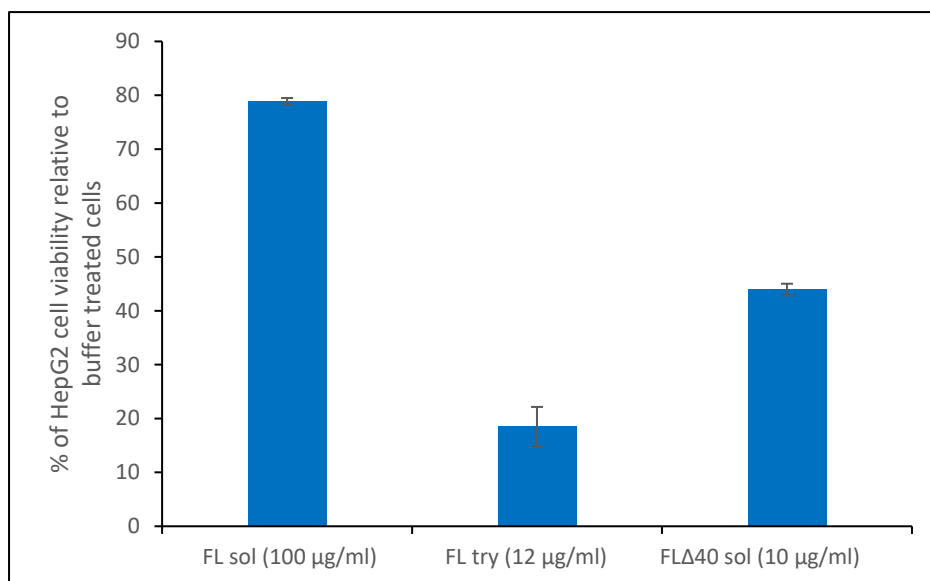


Figure 5: Evaluation of the level of toxicity of solubilised FLΔ40.

HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with solubilised FL (100 µg/ml), solubilised FLΔ40 (10 µg/ml) and trypsin activated FL (12 µg/ml). 24 hours later, cell viability was measured using CellTiter-Blue.

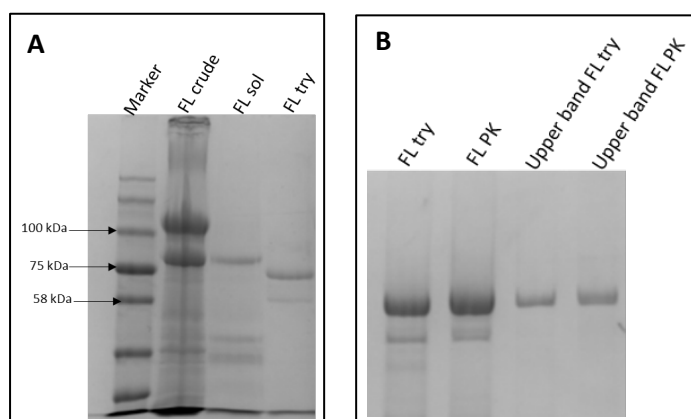


Figure 6: Purification of the upper band produced following Cry41Aa activation.

A: SDS-PAGE analysis of crude, solubilised and trypsin activated full length Cry41Aa; Crude full length Cry41Aa (FL) was solubilised for 1 hour at 37°C in 50 mM sodium carbonate (pH 10.5) in the presence of 5 mM DTT and activated with trypsin (1 mg/ml) for 1 hour at 37°C. Samples were then run on 7.5% SDS-PAGE gel. **B:** SDS-PAGE showing successful separation of the upper band from the mixture following Cry41Aa activation; selected ÄKTA purified fractions comprising only the upper band of trypsin and PK activated Cry41Aa were desalted, concentrated then run on 7.5% gel along with ÄKTA purified toxins which contain the two fragments.

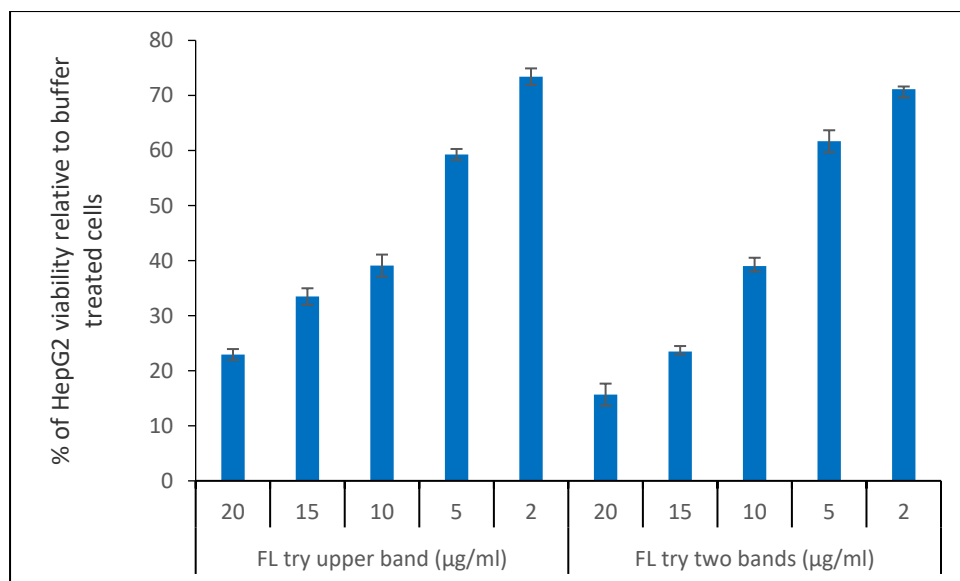


Figure 7: Evaluation of the level of toxicity of trypsin and PK activated Cry41Aa and the upper bands yielded post activation on HepG2 cell line.

HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with different concentrations of purified trypsin and PK activated Cry41Aa and the upper bands produced after activation. 24 hours later, cell viability was measured using CellTiter-Blue.

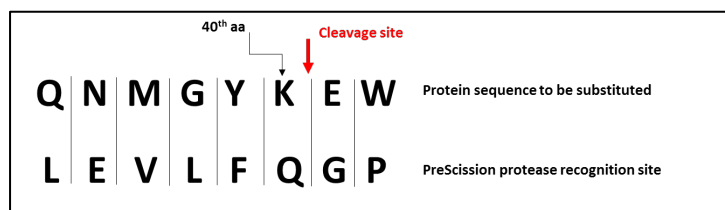


Figure 8: Schematic explanation of insertion of PreScission cleavage site in order to ensure cleavage at the 40th aa in the N-terminal region of Cry41Aa.

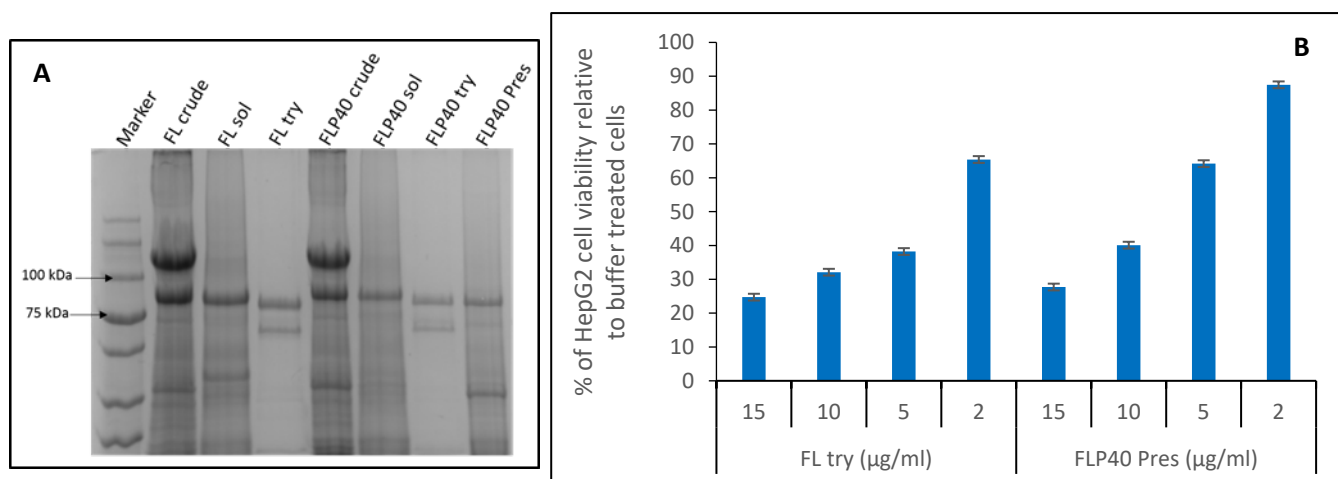


Figure 9: Role of C-terminal region of Cry41Aa in its cytotoxic activity.

A: SDS-PAGE analysis of FLP40 protein levels and sizes compared with Cry41Aa full length (FL); Crude FLP40 and Cry41Aa (FL) were solubilised for 1 hour at 37°C in 50 mM sodium carbonate (pH 10.5) in the presence of 5 mM DTT and activated with trypsin (1 mg/ml) for 1 hour at 37°C or with PreScission (130 µg/ml) for 16 hours at 4°C. The samples were then run on 7.5% SDS-PAGE gel. **B:** Evaluation of the level of toxicity of FLP40; HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with different concentrations of trypsin activated Cry41Aa (FL try) (15, 10, 5, 2 µg/ml) and PreScission activated FLP40 (FLP40 Pres) (15, 10, 5, 3, 2 µg/ml). 24 hours later, cell viability was measured using CellTiter-Blue.

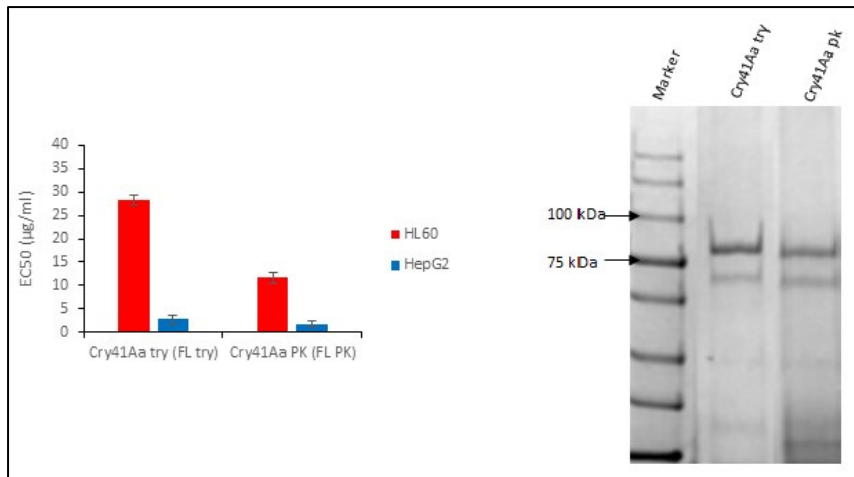


Figure 10: Comparison between the level of toxicity of trypsin and PK activated Cry41Aa full length on HepG2 and HL-60 cell lines. HepG2 and HL-60 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with different concentrations of purified trypsin and PK activated Cry41Aa. 24 hours later, cell viability was measured using CellTiter-Blue assay. The EC₅₀s were determined using SPSS software.

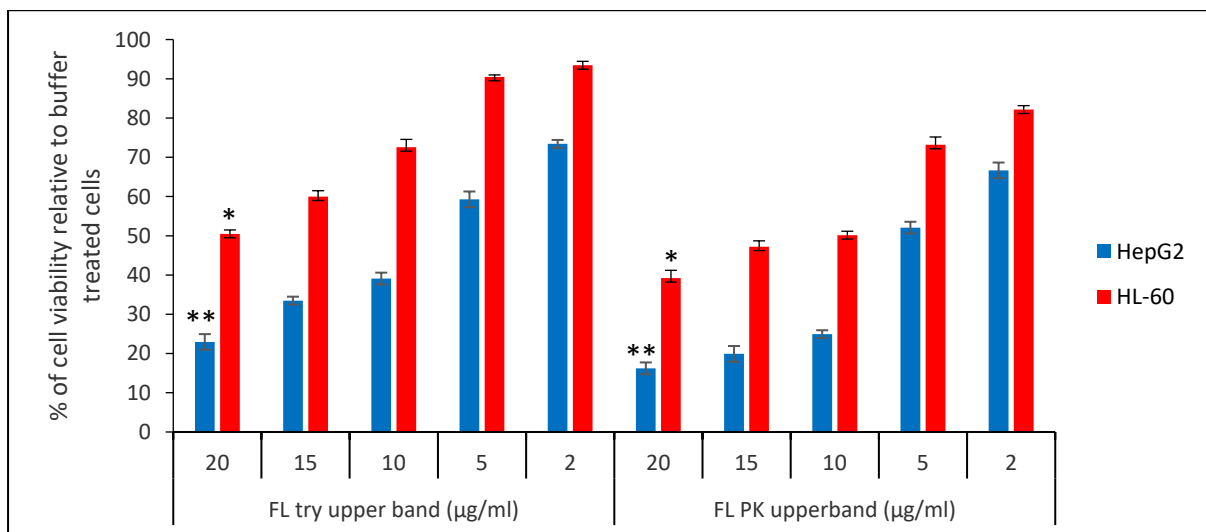


Figure 11: Evaluation of the level of toxicity of trypsin and PK activated Cry41Aa and the upper bands yielded post activation on HL-60 and HepG2 cell lines.

HL-60 and HepG2 cells were seeded at a density of 25×10^4 cells/ml. The next day the cells were treated with different concentrations of purified trypsin and PK activated Cry41Aa and the upper bands produced after activation. 24 hours later, cell viability was measured using CellTiter-Blue. Mean of three biological repeats; t-test * $p < 0.001$, ** $p < 0.04$ (p values were in the same range for all other concentrations used).

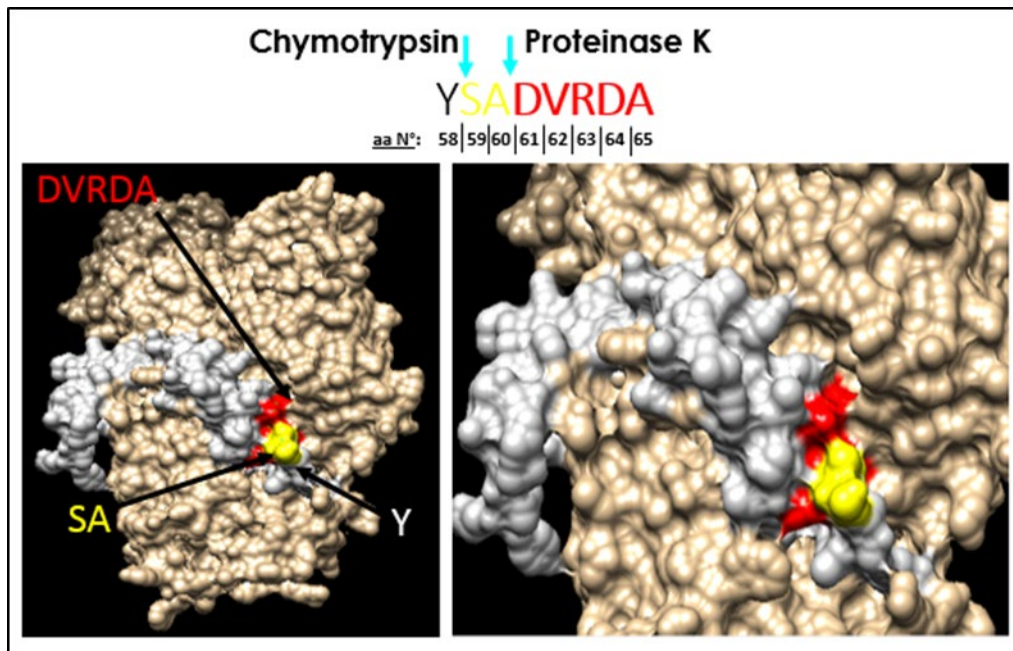


Figure 12: Diagrams showing the surface view of the structure of Cry41Aa modelled by Phyre2 and visualised by Chimera.
 The red part represents DVRDA hypothesized to correspond to a binding epitope, the yellow part represent Ser and Ala aa hypothesized to be interfering with binding and the grey part represents the N-terminal region containing the tyrosine (Y), chymotrypsin cleavage site.